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TITLE OF THE INVENTION

PROTEINS AND DNA RELATED TO SALT TOLERANCE IN PLANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit to U.S. provisional application No 60/194,648, filed on April 04, 2000, and incorporated herein by reference in its entirety.

STATEMENT OF FEDERALLY FUNDED RESEARCH

This invention was supported by the National Institutes of Health by Contract No. R01GM59138. The government may have certain rights to this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to proteins and nucleic acids related to salt tolerance in plants.

Description of the Background

Soil salinity is a major abiotic stress for plant agriculture. Sodium ions in saline soils are toxic to plants due to its adverse effects on K^+ nutrition, cytosolic enzyme activities, photosynthesis and metabolism (1, 2). Three mechanisms function cooperatively to prevent the accumulation of Na^+ in the cytoplasm, i.e. restriction of Na^+ influx, active Na^+ efflux and compartmentation of Na^+ in the vacuole (1). The wheat high-affinity K^+ transporter HKT1 functions as a Na^+-K^+ cotransporter, which confers low-affinity Na^+ uptake at toxic Na^+ concentrations (3). Thus HKT1 could represent one of the Na^+ uptake pathways in plant roots. The low-affinity cation transporter LCT1 from wheat may also mediate Na^+ influx into

plant cells (4). In addition, patch clamp studies have shown that non-selective cation channels play important roles in mediating Na^+ entry into plants (5). The Arabidopsis thaliana *AtNHX1* gene encodes a tonoplast Na^+/H^+ antiporter and functions in compartmentalizing Na^+ into the vacuole (6). Over-expression of *AtNHXl* enhances the salt tolerance of Arabidopsis plants (7).

No Na^+ efflux transporter has been cloned from plants. Plants do not appear to have a Na^+-ATPase at the plasma membrane (1). It is expected that proton motive force created by H^+-ATPases would drive Na^+ efflux from plant cells through plasma membrane Na^+/H^+ antiporters (8). Fungal cells contain both Na^+-ATPases and Na^+/H^+ antiporters at the plasma membrane. In the yeast *Saccharomyces cerevisiae*, plasma membrane Na^+-ATPases play a predominant role in Na^+ efflux and salt tolerance (9). In contrast, Na^+/H^+ antiporters are more important for Na^+ efflux and salt tolerance in the fungus *Schizosaccharomyces pombe* (10).

Recently, several *Arabidopsis sos* (for *s*alt *o*verly *s*ensitive) mutants defective in salt tolerance were characterized (11,12,13). The *sos* mutants are specifically hypersensitive to high external Na^+ or Li^+ and also unable to grow under very low external K^+ concentrations (13). Allelic tests indicated that the *sos* mutants define three *SOS* loci, i.e., *SOS1*, *SOS2* and *SOS3* (13). The *SOS3* gene encodes an EF-hand type calcium-binding protein with similarities to animal neuronal calcium sensors and the yeast calcineurin B subunit (14). In yeast, calcineurin plays a central role in the regulation of Na^+ and K^+ transport. Mutations in calcineurin B lead to increased sensitivity of yeast cells to growth inhibition by Na^+ and Li^+ stresses (15). The *SOS2* gene was recently cloned and shown to encode a serine/threonine type protein kinase (16). Interestingly, *SOS2* physically interacts with and is activated by *SOS3* (17). Therefore, *SOS2* and *SOS3* define a novel regulatory pathway for Na^+ and K^+ homeostasis and salt tolerance in plants. The *SOS3/SOS2* pathway has been predicted to control the expression and/or activity of ion transporters (17). However, the identities of the transporters regulated by this pathway are not known.

Among the three *SOS* loci, *SOS1* plays the greatest role in plant salt tolerance. Compared to *sos2* and *sos3* mutant plants, *sos1* mutant plants are even more sensitive to $Na^+$and Li^+ stresses (13). Double mutant analysis indicated that *SOS1* functions in the same pathway as *SOS2* and *SOS3* (12, 13). Thus, *SOS1* may be a target for regulation by the *SOS3/SOS2* pathway.

Accordingly, there remains a need in the art to isolate the *SOS1* gene and the protein

encoded thereby.

Furthermore, because of limited water supplies and the widespread use of irrigation, the soils of many cultivated areas have become increasingly salinized. In particular, modern agricultural practices such as irrigation impart increasing salt concentrations when the available irrigation water evaporates and leaves previously dissolved salts behind. As a result, the development of salt tolerant cultivars of agronomically important crops has become important in many parts of the world. For example, in salty soil found in areas such as Southern California, Arizona, New Mexico and Texas.

Dissolved salts in the soil increase the osmotic pressure of the solution in the soil and tend to decrease the rate at which water from the soil will enter the roots. If the solution in the soil becomes too saturated with dissolved salts, the water may actually be withdrawn from the plant roots. Thus the plants slowly starve though the supply of water and dissolved nutrients may be more than ample. Also, elements such as sodium are known to be toxic to plants when they are taken up by the plants.

Salt tolerant plants can facilitate use of marginal areas for crop production, or allow a wider range of sources of irrigation water. Traditional plant breeding methods have, thus far, not yielded substantial improvements in salt tolerance and growth of crop plants. In addition, such methods require long term selection and testing before new cultivars can be identified.

Accordingly, there is a need to increase salt tolerance in plants, particularly those plants which are advantageously useful as agricultural crops.

SUMMARY OF THE INVENTION

The present invention is based, in part, on the isolation of the *SOS1* locus through positional cloning. It is predicted to encode a transmembrane protein with similarities to plasma membrane Na^+/H^+ antiporters from bacteria and fungi. The results of the present invention suggest that a plasma membrane-type Na^+/H^+ antiporter is essential for plant salt tolerance. The steady state level of *SOS1* transcript is up-regulated by NaCl stress. The *sos2* mutation abolishes *SOS1* up-regulation in the shoot. In the *sos3* mutant, no *SOS1* up-regulation is found in the shoot or root. Therefore, *SOS1* gene expression under NaCl stress is controlled by the *SOS3/SOS2* regulatory pathway.

Accordingly, the present invention provides an isolated polynucleotide which encodes a protein comprising the amino acid sequence of SEQ ID NO:2.

In a preferred embodiment the polypeptide has Na+/H+ transporter activity.

In another preferred embodiment the polynucleotide comprises SEQ ID NO:1, polynucleotides which are complimentary to SEQ ID NO:1, polynucleotides which are at least 70%, 80% and 90% identical to SEQ ID NO:1; or those sequence which hybridize under stringent conditions to SEQ ID NO:1, the stringent conditions comprise washing in 5X SSC at a temperature from 50 to 68°C.

In another preferred embodiment the polynucleotides of the present invention are in a vector and/or a host cell. Preferably, the polynucleotides are in a plant cell or transgenic plant. Preferably, the plant is Arabidopsis thaliania or selected from the group consisting of wheat, corn, peanut cotton, oat, and soybean plant. In a preferred embodiment, the polynucleotides are operably linked to a promoter, preferably an inducible promoter.

In another preferred embodiment the present invention provides, a process for screening for polynucleotides which encode a protein having Na+/K+transporter activity comprising hybridizing the polynucleotide of the invention to the polynucleotide to be screened; expressing the polynucleotide to produce a protein; and detecting the presence or absence of Na+/K+transporter activity in said protein.

In another preferred embodiment, the present invention provides a method for detecting a nucleic acid with at least 70% homology to nucleotide SEQ ID NO:1, sequences which are complimentary to SEQ ID NO:1 and/or which encode a protein having the amino acid sequence in SEQ ID NO:2 comprising contacting a nucleic acid sample with a probe or primer comprising at least 15 consecutive nucleotides of the nucleotide sequence of Claim 1, or at least 15 consecutive nucleotides of the complement thereof.

In another preferred embodiment, the present invention provides a method for producing a nucleic acid with at least 70% homology to the polynucleotides of the present invention comprising contacting a nucleic acid sample with a primer comprising at least 15 consecutive nucleotides of the nucleotide sequence of Claim 3, or at least 15 consecutive nucleotides of the complement thereof.

In another preferred embodiment, the present invention provides a method for making SOS2 protein, comprising culturing the host cell carrying the polynucleotides of the invention for a time and under conditions suitable for expression of SOS2, and collecting the SOS2 protein.

In another preferred embodiment, the present invention provides a method of making

a transgenic plant comprising introducing the polynucleotides of the invention into the plant.

In another preferred embodiment, the present invention provides method of increasing the salt tolerance of a plant in need thereof, comprising introducing the polynucleotides of the invention into said plant.

In another preferred embodiment, the present invention provides an isolated polypeptide comprising the amino acid sequence in SEQ ID NO:2 or those proteins that are at least 70%, preferably 80%, preferably 90% and preferably 95% identity to SEQ ID NO:2. Preferably, the polypeptides have Na+/K+transporter activity.

BRIEF DESCRIPTION OF THE FIGURES

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings, wherein:

Fig. 1. Positional cloning of the *SOS1* gene. (A) Physical mapping of *SOS1*. All the SSLP markers shown except ngal 145 were developed in this study based on sequence information of the bacterial artificial chromosomes (BACs). The BAC contig was assembled based on information available at http://www.Arabidopsis.org/cgi-bin/maps, incorporated herein by reference. (B) Structure of the *SOS1* gene. Positions are relative to the initiation codon. Filled boxes indicate the open reading frame and lines between boxes indicate introns.

Fig. 2. Complementation of *sos1* by 35S-*SOS1*. Seven-day-old seedlings grown on MS agar medium were transferred to MS medium supplemented with 100 mM NaCl. The picture was taken after 10 days of treatment on the NaCl medium. Left, wild type plants (WT). Center, *sos1*-1 mutant plants. Right, transgenic *sos1*-1 plants containing the wild type *SOS1* gene under control of the CaMV 35S promoter. These plants did not show any difference when grown on MS medium without supplementation of NaCl.

Fig. 3. *SOS1* is predicted to encode a transmembrane protein. (A) The deduced amino acid sequence of *SOS1*. The 12 putative transmembrane domains (TM) are underlined. (B) Hydrophobicity plot of *SOS1*. The hydrophobicity values were calculated by the program Tmpred available at http://www.ch.embnet.org/software/TMPRED_form.html, incorporated herein by reference.

Fig. 4. *SOS1*, is similar to Na^+/H^+ antiporters. (A) Alignment of *SOS1* (accession number AF256224) with Na^+/H^+ antiporters NHEl from Chinese hamster (P48761) and NhaP from *Pseudomonas aeruginosa* (BAA31695). The sequences were aligned by the program ClustalW (http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html, incorporated herein by reference). Amino acids identical in at least two proteins are highlighted in black and conservative substitutions in grey. * indicates conserved residues that were substituted in *sos1* mutant alleles. (B) Phylogenetic analysis of *SOS1* and other representative Na^+/H^+ antiporters. Multiple sequence alignment was performed with ClustalW. The alignment is based on the N-terminal 450 amino acids of *SOS1*. Evolutionary distances were calculated by the Neighbor Joining method and the phylogenetic tree was drawn by the program Drawgram (http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html, incorporated herein by reference). The accession number and source of each of the other representative Na^+/H^+ antiporters are as follows: NHE1 (P19634), *Homo sapiens*; NHE2 (AAD41635), *Homo sapiens*; NHE3 (P48764), *Homo sapiens*; NHE4 (P26434), *Rattus norvegicus*; NIBS (AAC98696.1), *Homo sapiens*; NHE6 (NP_006350), *Homo sapiens*; NHA1 (NP_013239), *Saccharomyces cerevisiae*; NHX1 (NP 010744), *Saccharomyces cerevisiae*; AtNHXl (AAD16946.1), *Arabidopsis thaliana*; SOD2 (CAA77796.1), *Schizosaccharomyces pombe*; NhaA (P13738), *Escherichia coli*; NhaP (BAA31695.1), *Pseudomonas aeruginosa*.

Fig. 5. Diagrammatic representation of SOS1 structure. The diagram was drawn based on the prediction of hydrophobicity profile of *SOS1*. Putative transmembrane helices are shown as cylinders. The positions of mutations in *sos1* alleles are indicated.

Fig. 6. SOS1 expression is up-regulated by NaCl stress and is under control of the SOS3/SOS2 regulatory pathway. (A) *SOS1* expression is specifically up-regulated by NaCl stress in wild type Arabidopsis seedlings. (B) Up-regulation of *SOS1* expression in roots and shoots of wild type plants. (C) *SOS1* expression in *sos2*-1 mutant seedlings. (D) *SOS1* expression in *sos3*-1 mutant seedlings. The same RNA blots were hybridized successively with *SOS1*, RD29A and actin cDNA probes. Actin was used as loading control and RD29A as control for the stress treatments. C, control treatment.

Fig. 7. Nucleotide sequence of the SOS1 gene (Genbank accession number AF256224).

Fig. 8. Overexpression of SOS1 improves salt tolerance of Arabidopsis plants.

Transformed plants overexpressing SOS1 and control plants that were transformed with a vector only were compared for their salt tolerance. All plants were grown in soil on a long-day cycle (16 hours light, 8 hours dark). The first 13 days after germination the plants were watered with a diluted nutrient solution (1/8 MS) as needed. Starting on the 14th day, this solution was supplemented with NaCl. The supplementations consisted of four increasingly higher concentrations (50 mM, 100 mM, 150 mM, and 200 mM) of NaCl. The plants were treated for four days at each concentration, for a total of 16 days. On the 16th day the picture shown in the figure was taken.

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of molecular biology. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques, encompassed by the present invention. See, for example, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1982) and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989); Methods in Plant Molecular Biology, Maliga et al, Eds., Cold Spring Harbor Laboratory Press, New York (1995); Arabidopsis, Meyerowitz et al, Eds., Cold Spring Harbor Laboratory Press, New York (1994) and the various references cited therein.

The term "plant" includes whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. The class of plants which can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. Preferred plants include rice, corn, wheat, cotton, peanut, and soybean.

Thus, in one embodiment of the present invention, the salt tolerance of a plant can be enhanced or increased by increasing the amount of protein available in the plant, preferably by the enhancement of the SOS1 gene in the plant.

Thus, one embodiment of the present invention are plant cells carrying the polynucleotides of the present invention, and preferably transgenic plants carrying the isolated polynucleotides of the present invention.

As used herein, the term "enhancement" means increasing the intracellular activity of one or more enzymes in a plant cell and/or plant which are encoded by the corresponding DNA. Enhancement can be achieved with the aid of various manipulations of the bacterial cell. In order to achieve enhancement, particularly over-expression, the number of copies of the corresponding gene can be increased, a strong promoter can be used, or the promoter- and regulation region or the ribosome binding site which is situated upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same manner. In addition, it is possible to increase expression by employing inducible promoters. A gene can also be used which encodes a corresponding enzyme with a high activity. Expression can also be improved by measures for extending the life of the mRNA. Furthermore, enzyme activity as a whole is increased by preventing the degradation of the enzyme. Moreover, these measures can optionally be combined in any desired manner. These and other methods for altering gene activity in a plant are known as described, for example, in Methods in Plant Molecular Biology, Maliga et al, Eds., Cold Spring Harbor Laboratory Press, New York (1995).

A gene can also be used which encodes a corresponding or variant enzyme with a high activity. Preferably the corresponding enzyme has a greater activity than the native form of the enzyme, more preferably at least in the range of 5, 10, 25% or 50% more activity, most preferably more than twice the activity of the native enzyme.

In the context of the present Application, a polynucleotide sequence is "homologous" with he sequence according to the invention if at least 70%, preferably at least 80%, most preferably at least 90% of its base composition and base sequence corresponds to the sequence according to the invention. According to the invention, a "homologous protein" is to be understood to comprise proteins which contain an amino acid sequence at least 70 % of which, preferably at least 80 % of which, most preferably at least 90 % of which,

corresponds to the amino acid sequence which is encoded by the SOS1 gene (SEQ ID No.1), wherein corresponds is to be understood to mean that the corresponding amino acids are either identical or are mutually homologous amino acids. The expression "homologous amino acids" denotes those which have corresponding properties, particularly with regard to their charge, hydrophobic character, steric properties, etc.

Homology, sequence similarity or sequence identity of nucleotide or amino acid sequences may be determined conventionally by using known software or computer programs such as the *BestFit* or *Gap* pairwise comparison programs (GCG Wisconsin Package, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin 53711). *BestFit* uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2: 482-489 (1981), to find the best segment of identity or similarity between two sequences. *Gap* performs global alignments: all of one sequence with all of another similar sequence using the method of Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970). When using a sequence alignment program such as *BestFit*, to determine the degree of sequence homology, similarity or identity, the default setting may be used, or an appropriate scoring matrix may be selected to optimize identity, similarity or homology scores. Similarly, when using a program such as *BestFit* to determine sequence identity, similarity or homology between two different amino acid sequences, the default settings may be used, or an appropriate scoring matrix, such as *blosum45* or *blosum80*, may be selected to optimize identity, similarity or homology scores.

The present invention also relates to polynucleotides which contain the complete gene with the polynucleotide sequence corresponding to SEQ ID No. 1 or fragments thereof, and which can be obtained by screening by means of the hybridization of a corresponding gene bank with a probe which contains the sequence of said polynucleotide corresponding to SEQ ID No. 1 or a fragment thereof, and isolation of said DNA sequence.

Polynucleotide sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate those cDNAs or genes which exhibit a high degree of similarity to the sequence of the SOS1 gene.

Polynucleotide sequences according to the invention are also suitable as primers for polymerase chain reaction (PCR) for the production of DNA which encodes an enzyme having activity of a Na^+/H^+ transporter.

Oligonucleotides such as these, which serve as probes or primers, can contain more than 30, preferably up to 30, more preferably up to 20, most preferably at least 15 successive nucleotides. Oligonucleotides with a length of at least 40 or 50 nucleotides are also suitable.

The term "isolated" means separated from its natural environment.

The term "polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, and can denote an unmodified RNA or DNA or a modified RNA or DNA.

The term "polypeptides" is to be understood to mean peptides or proteins which contain two or more amino acids which are bound via peptide bonds.

The polypeptides according to invention include polypeptides corresponding to SEQ ID No. 2, particularly those with the biological activity of a Na^+/H^+ transporter, and also includes those, at least 70 % of which, preferably at least 80% of which, are homologous with the polypeptide corresponding to SEQ ID No. 2, and most preferably those which exhibit a homology of least 90 % to 95 % with the polypeptide corresponding to SEQ ID No. 2 and which have the cited activity.

The invention also relates to coding DNA sequences which result from SEQ ID No. 1 by degeneration of the genetic code. In the same manner, the invention further relates to DNA sequences which hybridize with SEQ ID No. 1 or with parts of SEQ ID No. 1. Moreover, one skilled in the art is also aware of conservative amino acid replacements such as the replacement of glycine by alanine or of aspartic acid by glutamic acid in proteins as "sense mutations" which do not result in any fundamental change in the activity of the protein, i.e. which are functionally neutral. It is also known that changes at the N- and/or C-terminus of a protein do not substantially impair the function thereof, and may even stabilise said function.

In the same manner, the present invention also relates to DNA sequences which hybridize with SEQ ID No. 1 or with parts of SEQ ID No. 1. Finally, the present invention relates to DNA sequences which are produced by polymerase chain reaction (PCR) using oligonucleotide primers which result from SEQ ID No. 1. Oligonucleotides of this type typically have a length of at least 15 nucleotides.

The terms "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a polynucleotide will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing).

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C. for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C., and a wash in 1X to 2X SSC (20X SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C., and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C., and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA--DNA hybrids, the Tm can be approximated from the equation of Meinkoth and Wahl, Anal. Biochem., 138:267-284 (1984): Tm =81.5oC.+16.6 (log M)+0.41 (%GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The Tm is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. Tm is reduced by about 1°C. for each 1% of mismatching; thus, Tm, hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with approximately 90% identity are sought, the Tm can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C

lower than the thermal melting point (Tm) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C. lower than the thermal melting point (Tm); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C. lower than the thermal melting point (Tm); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C. lower than the thermal melting point (Tm). Using the equation, hybridization and wash compositions, and desired Tm, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a Tm of less than 45 °C. (aqueous solution) or 32 °C. (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Current Protocols in Molecular Biology, Chapter 2, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (2000).

Thus, with the foregoing information, the skilled artisan can identify and isolated polynucleotides which are substantially similar to the present polynucleotides. In so isolating such a polynucleotide, the polynucleotide can be used as the present polynucleotide in, for example, increasing the salt tolerance of a plant.

One embodiment of the present invention is methods of screening for polynucleotides which have substantial homology to the polynucleotides of the present invention, preferably those polynucleotides encode a protein having Na^+/H^+ transporter activity.

The polynucleotide sequences of the present invention can be carried on one or more suitable plasmid vectors, as known in the art for plants or the like.

In one embodiment, it may be advantageous for propagating the polynucleotide to carry it in a bacterial or fungal strain with the appropriate vector suitable for the cell type. Common methods of propagating polynucleotides and producing proteins in these cell types are known in the art and are described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1982) and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989).

SPECIFIC EMBODIMENTS OF THE INVENTION

MATERIALS AND METHODS

Genetic Mapping

sos1 mutant plants in the Columbia (Col) background were crossed to wild type plants of the Landsberg (Ler) ecotype. *sos* mutants were selected from the segregating F2 population by the root-bending assay (11). Genomic DNA from 1663 individual mutant F2 plants was analyzed for cosegragation with SSLP markers. For the fine mapping of *SOS1*, 7 SSLP markers were developed based on genomic sequences of the BAC clones at the top of chromosome 2. The primer pairs for the SSLP markers that are polymorphic between Col and Ler are as follows:

T20F6-1-F: 5'-GGATGATGATCGATTCGGAT-3'

T20F6-1-R: 5'-ATCTGACTCATAGGATATCG-3'

ngal 145-F: 5'-CCTTCACATCCAAAACCCAC-3'

ngal 145-R: 5'-GCACATACCCACAACCAGAA-3'

F5O4-3-F: 5'-GAATGTTTTGAAGGATATCTCAG-3'

F5O4-3-R: 5'-GAAAAATGGAGCACGAAATAAGC-3'

F14H20-3-F: 5'-CCCGAGATTAATACACAATC-3'

F14H20-3-R: 5'-GCAGATTATGTAATTGTGACC-3'

T23K3-1-F: 5'-TCGTGTTTACCGGGTCGGAT-3'

T23K3-1-R: 5'-TGATGAGAATCTTAGCGAGC-3'

CCC-1-F: 5'-TGGTAAGACCAAATTACACTC-3'

CCC-I-R: 5'-CGTAATTAAAATGTGTTAAACCG-3'

F10A8-1-F: 5'-AACCGCATAGTACAATGCAG-3'

F10A8-1-R: 5'-CGGTAAAGATCAACTAATAACG-3'

F23H14-3-F :5'-AACGGAAACGGCAACTAGAC-3'

F23H14-3-R: 5'-ACCCTAAATGTTTCGATTCG-3'

DNA Sequencing

To determine the nucleotide sequence of *SOS1* gene in *sos1* mutant alleles, synthetic oligonucleotide primers were made that would enable sequencing of the entire gene. Overlapping fragments encompassing the entire *SOS1* gene were PCR amplified by using these primers. The amplified products were sequenced on both strands. To avoid errors due to PCR, three independent PCR samples were mixed and batch sequenced.

Isolation of cDNA

cDNA containing the complete *SOS1* open reading frame was obtained by reverse transcription (RT)-PCR amplification. RNA, from salt-treated Col wild type plants was used as template for the RT-PCR. Three overlapping cDNA fragments obtained from RT-PCR were mixed as the template to amplify a full length cDNA which was then cloned into pCR-Blunt II-TOPO Vector (Invitrogen).

Plant Transformation and Complementation Test

SOS1 cDNA containing the entire open reading frame was cloned into the *XbaI* and *SacI* sites of pBI121. The construct was introduced into *Agrobacterium* GV3101 strain, and the resulting bacteria were used to transform *sos1-1* mutant plants by vacuum infiltration (18). Kanamycin resistant T2 transgenic plants were selected and subjected to complementation tests on MS agar medium supplemented with 100 mM NaCl.

RNA Analysis

Arabidopsis seedlings were grown on MS agar medium under continuous light (11). Ten-day-old seedlings were used for different treatments. For salt treatment, the seedlings

were transferred onto a Whatman filter paper soaked with 3 mM NaCl and treated for 5 h. For ABA treatment, the seedlings were sprayed with 10 μM ABA and kept for 3 h. For cold treatment, the seedlings on MS agar medium were incubated at 0°C for 24 h. To determine gene expression in root and shoot separately, seedlings were grown on agar surface in vertical plates for 10 days, treated with NaCl by immersing the roots in MS nutrient solution supplemented with 200 mM NaCl for 6 h. RNA extraction and Northern analysis were carried out as described (13).

RESULTS

Positional Cloning of *SOS1*

By examining several PCR based molecular markers, we found that the SSLP marker ngal 145 near the top of chromosome 2 is closely linked to the *sos1* mutation. Seven new SSLP markers were then developed based on the genomic sequence of BAC clones at the top of chromosome 2. Fine mapping using these markers delimited *SOS1* to about 70 kb region between the molecular markers T23K3-1 and F14H20-3 (Fig. 1 A). Candidate genes in this region were amplified from soil mutants and sequenced. The sequence analysis revealed that a putative gene, F14H20.5, contains a 2 bp deletion in the *sosl-13* mutant allele generated by fast neutron bombardment. Further analyses showed that all *sos1* alleles contain mutations in this putative gene and each mutation causes a change in the amino acid sequence (Table 1). Furthermore, expression of this candidate gene under control of the CaMV 35S promoter complemented the salt-hypersensitive phenotype of *sosl-1* mutant plants (Fig. 2). When *sos1 -1* mutant seedlings were treated with 100 mM NaCl, their growth was arrested. In these mutant plants, older leaves became chlorotic while young leaves became dark in color. In contrast, *sos1-1* mutant plants containing the 35S-*SOS1* transgene could grow, and remained green under 100 mM NaCl treatment, as did the wild type plants. Based on these results, we conclude that this putative gene is *SOS1*.

SOS1 Encodes a Putative Na^+/H^+ Antiporter

The *SOS1* open reading frame was determined by sequencing several overlapping

cDNAs obtained from young *Arabidopsis* seedlings by reverse transcriptase polymerase chain reaction. Comparison with the genomic sequence revealed that *SOS1* has 22 introns and 23 exons (Fig. 1B). *SOS1* is predicted to encode a polypeptide of 1146 amino acid residues (Fig. 3A) with a theoretical molecular mass of 127 kDa. Hydrophobicity plot analysis showed that the N-terminal portion of *SOS1* is highly hydrophobic and has 12 predicted transmembrane domains (Fig. 3B). Database searches revealed substantial similarities between the transmembrane region of *SOS1* and Na^+/H^+ antiporters of animal or microbial origins (Fig. 4A). Over a stretch of 342 amino acid residues (113-443), *SOS1* has 26% identity and 45% similiarity with NHE1 from Chinese hamster (19). The highest sequence similarities for *SOS1* are with the "eucaryotic" type Na^+/H^+ antiporters from bacteria, of which only NhaP from *Pseudomonas aeruginosa* has been functionally characterized (20). *SOS1* exhibits 31% identity and 48% similarity with the NhaP sequence over a stretch of 289 amino acids (131-408 in *SOS1*). The C-terminal portion of *SOS1* is hydrophilic and predicted to reside in the cytoplasm (Fig. 5). The long hydrophilic carboxyl-terminal tail makes *SOS1* the largest Na^+/H^+ antiporter sequence known to date. No similarities were Found between the *SOS1* tail region and other amino acid sequences in the GenBank database.

Phylogenetic analysis showed that *SOS1* clusters with plasma membrane Na^+/H^+ antiporters such as *SOD2*, NHa1, NhaA and NhaP (Fig. 4B). *SOD2* and NHA1 function on the plasma membrane of *S. pombe* and *S. cerevisiae*, respectively, to export Na^+ from cytosol to the extracellular space (21, 22, 23). NhaA and NhaP are Na^+/H^+ antiporters that function in Na^+ efflux in *E. coli* and *P. aeruginosa*, respectively (20, 24). *SOS1* is more distantly related to a cluster of orgaruellar Na^+/H^+ antiporters such as AtNHX1,. NHXl or NHE6 (Fig. 4B). AtNHXl functions on the tonoplast to compartmentalize Na^+ into the vacuole of *Arabidopsis* cells (6, 7). NHX1 plays a role in transporting Na^+ to the yeast prevacuolar compartment (25, 26). The animal Na^+/H^+ antiporter NHE6 has been reported to have a mitochondrial localization (2'7). SOS1 does not cluster with plasma membrane Na^+/H^+ antiporters from animals, which function in mediating Na^+ influx (28). These results suggest that SOS1 is distinct from vacuolar Na^+/H^+ antiporters, and may function at plant cell plasma membrane to mediate Na^+ efflux.

Analysis of *sosl* Mutant Alleles Reveals Several Residues and Regions Essential for SOS1

Function

The *SOS1* gene was amplified from thirty-two independent *sos1* mutant lines (13) and sequenced to determine the molecular basis of each mutation. Several mutant lines were found to harbor identical mutations (Table 1). Five of the fast neutron alleles result in relatively large deletions and were not assigned specific allele designations because the boundaries of the deletions are not known. Analysis of the various *sos1* mutations reveals several amino acid residues and regions essential for SOS1 function. The *sos1*-3 and *sos1*-12 alleles contain single amino acid substitutions in the membrane spanning region (Fig. 5). Both mutations affect residues that are conserved in all antiporters (Fig. 4A) and presumably abolish SOS1 antiport activity. Two other single amino acid substitution mutations (i.e. *sos1*-8 and *sos1*-9) one found in the hydrophilic tail region (Fig. 5). The *sos1*-10 allele was obtained from T-DNA mutagenesis and contains a 7-bp deletion that causes a frameshift that truncates the last 40 amino acids from the C-terminus of SOS1 (Fig. 5). Similarly, *sos1*-2 and *sos1*-6 mutations also truncate the cytoplasmic tail of *SOS1* (Fig. 5). These and other mutations that do not affect the transmembrane region reveal an essential role of the tail region for *SOS1* function. Like the hydrophilic tail of animal NHE1 antioporters (29), the tail of *SOS1* may interact with various regulators of antiport activity. As such, these mutations likely disrupt interaction between SOS1 and its regulators.

SOS1 expression Is Up-regulated Specifically by Salt Stress

To examine the expression of *SOS1* gene under stresses, RNA gel blot analysis was performed, *SOS1* mRNA was detected without stress treatment but was significantly up-regulated by salt stress (Fig. 6A). Consistent with its specific role in Na^+ tolerance, *SOS1* gene expression was not up-regulated by cold stress or ABA (Fig. 6A). In comparison, the RD29A gene was induced by ABA, cold as well as salt stresses. *SOS1* mRNA was more abundant in roots than in shoots. In both roots and shoots, *SOS1* expression was up-regulated by NaCl stress (Fig. 6B).

SOS1 Up-regulation Is Controlled by the SOS3/SOS2 Pathway

To determine whether NaCl up-regulation of *SOS1* is under control of the SOS3/SOS2 regulatory pathway, SOS] expression in *sos2*-l and *sos3*-1 mutant plants was analyzed. In the

sos2 mutant. *SOS1* was up-regulated by NaCl stress in the root but not in the shoot (Fig. 6C). In *sos3* plants, no *SOS1* up-regulation was seen in either the root or shoot (Fig. 6D). These results show that *SOS1* expression is regulated at least in part by the SOS3/SOS2 pathway.

DISCUSSION

SOS1 is a genetic locus that was previously identified as essential for plant salt tolerance (11). Mutations in *SOS1* render *Arabidopsis* plants extremely sensitive to high Na^+ or low K^+ environment (11, 13). In order to understand how the *SOS1* gene functions in salt tolerance, it was necessary to clone this gene. Even though several *sos1* mutant lines were recovered from a T-DNA insertion population, the T-DNA did not co-segregate with the *sos1* mutant phenotype (13). Therefore, a map-based strategy had to be utilized to clone the *SOS1* gene. Fine genetic mapping narrowed the search of *SOS1* to a very short region of chromosome 2. The fine mapping of *SOS1* was made possible by the several molecular markers we have developed and the large number of recombinant chromosomes examined. Several candidate genes in the region where *SOS1* is mapped were sequenced to identify the *sos1* mutation. One of the candidate genes was found to contain a mutation in every *sos1* mutant allele. Further confirmation that this candidate is indeed *SOS1*, came from genetic complementation test.

The *SOS1* protein is predicted to have 12 transmembrane domains in its N-terminal part. Throughout this transmembrane region, *SOS1* shows substantial sequence similarities with Na^+/H^+ antiporters from microbes and animals. The sequence similarities combined with the Na^+ hypersensitive phenotype of *sos1* mutant plants strongly indicate that *SOS1* is a Na^+/H^+ antiporter. Phylogenetic analysis showed that *SOS1* is more closely related to plasma membrane Na^+/H^+ antiporters from microorganisms than to the vacuolar antiporters from either plants or fungi. This suggests that *SOS1* is a plasma membrane Na^+/H^+ antiporter in *Arabidopsis*, As such, *SOS1* is expected to function in exporting Na^+ from the cytosol to the extracellular space, to prevent rapid accumulation of Na^+ in the cytoplasm.

SOS1 is predicted to have a cytoplasmic tail of approximately 700 amino acids in length. Sequence analysis of the multitude of *sos1* mutant alleles revealed that both the tail and transmembrane regions of *SOS1* are necessary for its function in plant salt tolerance. The

sos1-3, *sos1*-8, *sos1*-9 and *sos1*-12 mutations each causes a single amino acid substitution in the *SOS1* protein. Two of these substitutions occur in the transmembrane region and the other two in the tail. These four residues are clearly critical for *SOS1* function. The data presented herein on the *sos1* mutant lesions provide a wealth of information that will be valuable for detailed structure-function analysis.

SOS1 gene expression is up-regulated by NaCl stress. This is consistent with its role in Na^+ tolerance. It has been known that NaCl stress also up-regulates the expression of genes encoding plasma membrane H^+-ATPases (30). Increased H^+-ATPase expression would provide a greater proton motive force that is necessary for elevated Na^+/H^+ antiporter activity.

The *SOS3* calcium sensor physically interacts with the *SOS2* protein kinase (17). In the presence of calcium, *SOS3* activates *SOS2* kinase activity. The *SOS3-SOS2* kinase complex represents a regulatory pathway that specifically controls Na^+ and K^+ homeostasis and plant salt tolerance. Results presented in this paper suggest that one output of this pathway is the up-regulation of *SOS1* expression under NaCl stress. The *sos3* mutation abolishes *SOS1* up-regulation in both the root and shoot. In the *sos2* mutant, *SOS1* up-regulation in the shoot but not in root was disrupted. The fact that *SOS1* expression is still up-regulated in the root of *sos1* mutant indicates that there may be a functionally redundant root-specific *SOS2*-like kinase(s). The regulation of *SOS1* gene expression by the *SOS2/SOS3* pathway is consistent with previous genetic evidence suggesting that *SOS1* functions in the same pathway as *SOS2* and *SOS3* (12, 13).

SOS1 is essential for the homeostasis of both $Na^+$and K^+. Under NaCl stress, *sos1* mutant plants accumulate less Na^+ as well as less W (11, 31). *SOS1* gene expression is concentrated in cells surrounding the xylem, suggesting that *SOS1* may function in loading Na^+ into the xylem for long distance transport (our unpublished data). A xylem loading function of *SOS1* would be consistent with *sos1* mutant plants accumulating less Na^+. Preferential expression of *SOS1* at the symplast/xylem boundary would also help explain the K^+ transport defect of *sos1* mutant plants. It is well known that H^+ and Na^+ transport is closely linked at the xylem/symplast interface (32). The effect of *SOS1* on K^+ transport might be through its effect on W gradient across the cell membrane of stellar cells. For example, a K^+-H^+ symporter activity could be coupled with *SOS1* via H^+ cycling and such a symporter

may be required for high affinity W transport into the xylem. It is also possible that a K^+/Na^+ symporter is coupled with *SOS1* via Na^+ cycling.

Table 1. Molecular basis of *sos1* mutations.

Mutant line	Allele	Mutagen	Nucleotide change	Protein change
ssr1, Icss-3, Icss1-18	*sos1-1*	EMS	▲14bp, 1330-1343	frameshift
ss1-6, ss3-13	*sos1-2*	EMS	C5410-T	stop
ss1-16, Icss1-24	*sos1-3*	EMS	C2520-T	Arg-365-Cys
IIcss1-13, IIcss1-22	*sos1-4*	EMS	G-2480-A	stop
Icss1-10	*sos1-5*	EMS	G2766-A	splicing junction
Icss1-25	*sos1-6*	EMS	G3652-A	stop
IIcss1-59, css1-61	*sos1-7*	EMS	▲lbp, 4539	frameshift
Icss2-21	*sos1-8*	EMS	G-4594-A	Gly-777-Glu
Icss2-7	*sos1-9*	EMS	G-4615-A	Gly-784=Asp
tss2-1, p2901-3503 2-1	*sos1-10*	T-DNA	▲63bp, 2792-2854	splicing junction
P800 1-2, p800 1-3	*sos1-11*	T-DNA	▲7bp, 5953-5959	frameshift
FN50css2-3, FN50css3-22, FN75css1-24, FN75css1-14, FN75css3-18	*sos1-12*	fast neutron	G-668-A	Gly-136-Glu

Mutant line	Allele	Mutagen	Nucleotide change	Protein change
FN50css2-9, FN75css1-22, FN75css1-23	*sos1-13*	fast neutron	▲2bp, 5149-5150	frameshift
FN50css1-8, FN50css3-3, FN75css1-17, B46, B47		fast neutron	Whole gene deletions	

REFERENCES

1. Niu, X., Bressan R. A., Hasegawa, P, M. & Pardo, J. M. (1995) *Plant Physiol.* 109, 735-742.

2. Jacoby, B. (1999) in Handbook of Plant and Crop Stress. ed. Pessarakli M. (Marcel Dekker, New York), pp97-123.

3. Rubio, F., Gassman, W. & Schroeder, J. I. (1995) *Science* 270, 1660-1663.

4. Schachtman, D. P., Kumar, R., Schroeder, J.I. & Marsh, E. L. (1997) *Proc. Natl. Acad Sci.* U.S.A. 94, 11079-11084.

5. Amtmann, A. & Sanders, D. (1998) *Adv. Bot Res.* 29, 76-112.

6. Gaxiola R. A., Rao, R., Sherman, A., Grisafi, P., Alper, S. L. & Fink, G. R. (1999) *Proc. Natl. Acad. Sci.* U.S.A. 96, 1480-1485.

7. Apse, M. P., Aharon, G. S., Snedden, W. A. & Blumwald E. (1999) .*Science* 285, 1256-1258.

8. Schachtman, D. P. & Liu, W. (1999) *Trends Plant Sci.* 4, 281-287.

9. Haro, R., Garciadeblas, B. & Rodriguez-Navarro, A. (1991) *FEBS Lett.* 291, 189-191.

10. Jia, Z., McCullough, N., Martel, R., Hemmingsen, S. & Young, P. G. (1992) *EMBO J.* 11, 1631-1640.

11. Wu, S. -J., Lei, D. & Zhu, J. -K. (1996) *Plant Cell* 8, 617-.627.

12. Liu, J. & Zhu, J. -K. (1997) , *Proc. Natl. Acad Sci.* U.S.A. 94, 14960-14964.

13. Zhu, J. K., Liu, J. & Xiong, L. (1998) *Plant Cell* 10, 1181-1191.

14. Liu, J. & Zhu, J. -K. (1998) *Science* 280, 1943-1945.

15. Mendoza, I., Rubio, F., Rodriguez-Navarro, A. & Pardo, J. M. (1994) *J. Biol. Chem.* 269, 8792-8796.

16. Liu, J., Ishitani, M., Halfter, U., Kim, C. -S. & Zhu, J. -K. (2000) Proc. *Natl. Acad Sci. USA* (in press).

17. Halfter, U., Ishitani, M. & Zhu, J. -K. (2000) *Proc. Natl. Acad Sci, U.S.A.* (in press).

18. Bechtold, N., Ellis, J. & Pelletier, G. (1993) *CR Acad. Sci.* (Paris) 316, 1194-1199.

19. Counillon, L. & Pouyseegur, J. (1993) *Biochim. Biophys. Acta* 1172, 343-345

20. Utsugi, 3., Inaba, K., Kuroda, T., Tsuda, M. & Tsuchiya, T. (1998) *Biochim Biophys. Acta* 1398, 330-334,

21. Hahnenberger, K. M., Jia, Z. & Young, P. C. (1996) *Proc. Natl. Acad Sci. USA.* 93, 5031-5036.

22. Dibrov, P., Smith, J. J., Young, P. G. & Fliegel, L. (1997) *FEBS Lett.* 405, 119-124.

23. Prior, C., Potier, S., Souciet, J. L. & Sychrova, H. (1996) *FEBS Lett.* 387, 89-93.

24. Padan, E., Maisler, N., Taglicht, D., Karpel, R. & Schuldiner, S. (1989) *J. Biol. Chem.* 264, 20297-20302.

25. Nass, R., Cunningham, K. W. & Rao, R. (1997) *J. Biol. Chem.* 272, 26143-26152.

26. Nass, R. & Rao, R. (1998) *J. Biol. Chem.* 273, 21054-21060.

27. Numata, M., Petrecca, K., Lake, N. & Orlowski, J. (1998) *J. Biol. Chem.* 273, 6951-5959.

28. Orlowski, J. & Grinstein, S. (1997) *J. Biol. Chem.* 272, 22373-22376.

29. Silva, N. L., Haworth, R. S., Singh, D. & Fliegel L. (1995) *Biochem.* 34,10412-10420.

30. Niu, X., Narasimhan, M. L., Salzman, R. A., Bressan, R. A. & Hasegawa, P. M. (1993) *Plant Physiol.*143, 713-718.

31. Ding, L. & Zhu, J. -K. (1997) *Plant Physiol.* 113, 795-799.

32. Lacan, D. & Durand, M. (1996) *Plant Physiol.* 110, 705-711.